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ORIGINAL RESEARCH

Antitumor and antibacterial properties of virally encoded cationic sequences

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Objective: The objective of this study was to test our Viral Quinta Columna Strategy (VQCS), a new biological hypothesis predicting that specific multifunctional virally encoded cationic domains may have the capacity to penetrate human cells and interact with PP2A proteins to deregulate important human intracellular pathways, and may display LL37 cathelicidin-like antagonistic effects against multiple pathogens such as bacteria or viruses.

Methods: We comparatively analyzed the host defense properties of adenodiaphorins and of some specific cationic sequences encoded by different viruses using two distinct biological models: U87G, a well-characterized cell tumor model; and a group B *Streptococcus agalactiae* NEM316 ΔdltA, highly sensitive to LL37 cathelicidin.

Results: We found that the adenovirus type 2 E4orf4 is a cell-permeable protein containing a new E4orf4$_{64–95}$ protein transduction domain, named large adenodiaphorin or LadD$_{64–95}$. Interestingly, the host defense LL37 peptide is the unique cathelicidin in humans. In this context, we also demonstrated that similarly to LL37 LadD$_{64–95}$, several virally encoded cationic sequences including the C-terminus HIV-1 89.6 Vpr$_{77–92}$, shorter adenodiaphorins AdD$_{67–84}$/AdD/$_{69–84}$/AdD$_{69–83}$, as well as HIV-2 Tat$_{67–90}$ and JC polyomavirus small t$_{115–134}$, displayed similar toxicity against Gram-positive *S. agalactiae* NEM316 ΔdltA strain. Finally, LadD$_{64–95}$, adenodiaphorin AdD$_{67–84}$, AdD$_{69–84}$, and LL37 and LL$_{17–32}$ cathelicidin peptides also inhibited the survival of human U87G glioblastoma cells.

Conclusion: In this study, we demonstrated that specific cationic sequences encoded by four different viruses displayed antibacterial activities against *S. agalactiae* NEM316 ΔdltA strain. In addition, HIV-1 Vpr$_{71–92}$ and adenovirus 2 E4orf4$_{64–95,}$ two cationic penetrating sequences that bind PP2A, inhibited the survival of U87G glioblastoma cells. These results illustrate the host defense properties of virally encoded sequences and could represent an initial step for future complete validation of the VQCS hypothesis.

Keywords: cationic sequences, PP2A, cancer, viruses, bacteria

Introduction

Protein transduction domains (PTDs) and derived cell-penetrating peptides (cpps) are small peptide sequences derived from the few proteins that are naturally able to penetrate cells.[1–3] Furthermore, cpps usually contain short sequences with a positive charge resulting from several lysine and arginine residues, and are able both to deliver themselves and to deliver large micromolecules.[4] In addition, some cationic anti-microbial peptides (CAMPs), that have some similar physicochemical properties to cpps, can also have cell-penetrating properties, suggesting that they could be highly efficient therapeutic tools.[5,6] CAMPs, such as the unique anti-microbial human cathelicidin LL37 peptide, are naturally produced by the innate immune system and mediate a widespread anti-microbial activity against Gram-positive and

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Gram-negative bacteria such as *Staphylococcus aureus* and *Escherichia coli*, and also against fungi and enveloped viruses.[7,8] Conversely, Gram-positive bacteria have evolved the ability to increase their positive surface charge through D-alanylation of teichoic acid, thus gaining resistance to CAMPs.[9] In addition, both LL37 and its C-terminal fragment $LL_{17–32}$ (also known as FK16) exhibit cytotoxicity against distinct tumor cells.[7,10]

The PP2A family of serine/threonine protein phosphatases, a major target for cationic sequences, is critically involved in the regulation of numerous intracellular pathways.[11] In addition, various viruses encode specific proteins that interact with PP2A holoenzymes to specifically deregulate the intracellular pathways of their hosts.[12] In this regard, two distinct small viral cationic proteins, HIV-1 Vpr and adenovirus type 2 (ad2) E4orf4, interact with a trimeric PP2A holoenzyme ABC, named $PP2A_1$, to specifically induce p53-independent cell death.[13,14]

The C-terminus sequence of HIV-1 Vpr is a multifunctional domain with cell-penetrating, PP2A-mediated cell death and bactericidal anti-*E. coli* effects.[13,15] Similarly to HIV-1 $Vpr_{77–92}$, the ad2 $E4orf4_{64–95}$ ($LadD_{64–95}$) sequence contains residues required for PP2A binding, nuclear localization, and cell death.[14,16]

The Viral Quinta Columna Strategy (VQCS) is a new biological hypothesis that is based on combinatorial physical and biological properties, including cell penetration, PP2A interaction, and LL37-like host defense effects, that could be mediated by specific virally encoded cationic domains.[17] Consistent with this hypothesis, in this study we found that E4orf4 large adenodiaphorin ($LadD_{64–95}$) penetrating sequence, and cathelicidin active LL37 and $LL_{17–32}$ peptides inhibit survival of human U87G glioblastoma cells. In addition, similarly to LL37, some virally encoded cationic domains, such as HIV-1 $Vpr_{77–92}$, $LadD_{64–95}$, $AdD_{67–84}$, $AdD_{69–84}$, $AdD_{69–83}$, HIV-2 $Tat_{67–90}$, and polyoma JC small $t_{115–134}$ antigen, displayed similar toxicities against *Streptococcus agalactiae* NEM316 ΔdltA strain, a powerful Gram-positive bacterial model.[18]

Materials and methods

Cells

We used human glioblastoma U87G (kindly provided by Pr Marie Dutreix, Curie Institute, Orsay, France) and dermal human primary fibroblasts (DHF; Tebu-bio: https://www.tebu-bio.com/cms/743/.html) that had previously been approved by the Institut Pasteur, our institutional review board.

Peptides

Chemical solid-phase peptide synthesis of 15 NH_2-biotinylated peptides (listed in Table 1) was commercially realized by the French Proteogenix company at >95% purity (for profile see website: https://www.proteogenix.science/company/). NH_2-biotinylated peptides (Proteogenix) were prepared by solid-phase peptide synthesis, dissolved in DMSO, and stored at −20°C pending use. Full-length biotinylated E4orf4 polypeptide (sequence identification number P03240) was dissolved in methanol (60 μM) according to the manufacturer's recommendation.

Kits and reagents

We used streptavidin horseradish peroxidase (HRP) conjugate (Euromedex, Strasbourg, France), 3,3′-diaminobenzidine tetrahydrochloride (DAB) of the DAB Substrate Kit for Peroxidase (Vector Laboratories, Les Ulis, France), cOmplete™, Mini, EDTA-free Protease Inhibitor Cocktail Protease Inhibitor Cocktail Tablets (Roche, Meylan, France), and O-phenylenediamine dihydrochloride (OPD) tablets (Sigma Chemical Co, St Louis, MO, USA).

Quantification of peptide internalization

As previously described,[11] before incubation, the peptides were pre-incubated for 1 hour with streptavidin–HRP conjugate in a 4/1 ratio. Cells at 80% confluence were incubated with different concentrations of peptide in 24-well plates. After 6 hours, cells were washed twice in PBS, trypsinized (Trypsin EDTA; Invitrogen, Les Ulis, France), harvested in 1 mL of PBS, and counted. Cells were lysed in 300 μL of 0.1 M Tris (pH 7.4) and 0.5% Nonidet P-40 buffer for 10 minutes on ice. A total of 50 μL of cell lysate was mixed with 50 μL of OPD buffer (51.4 mM Na_2HPO_4 and 24.3 mM citric acid), then mixed with 100 μL of OPD solution (one OPD tablet; Sigma) in 100 mL of OPD buffer to which 40 μL of 30% hydrogen peroxide was added just before use. After 10–20 minutes, the reaction was stopped by adding 100 μL of 1 M HCl, and optical density (OD) was read at 490 nm. The assays were performed in triplicate. We used Gen5 detection software (BioTek, Colmar, France) for data capture and export into Excel, and Microsoft Excel software 2016 for macOS for statistical analyses in histograms with error bars indicating the SD.

Table 1 List of peptides

Origin	Acronym	Sequences
HIV-1	$Tat_{47–57}$	YGRRKKRRQRRR
	$Vpr_{71–92}$	HFRIGCRHSRIGIIQQRRTRNG
Adenovirus 2	E4orf4	MVLPALPAPPVCDSQNECVG WLGVAYSAVVD VIRAAAHEGVYIEPEARGRLDALREWIYYNYY TERAKRR DRRRRSVCHA RTWFCFRKYD YV RRSIWHDT TTNTISVVSAHSVQ
	$E4orf4\Delta_{(64–95)}$	MVLPALPAPPVCDSQNECVG WLGVAYSAVVD VIRAAAHEGVYIEPEARGRLDALREWIYYNYY SIWHDTTTNTISVVSAHSVQ
	$LadD_{64–95}$	TERAKRRDRRRRSVCHARTWFCFRKYDVRRS
	LadDmut	TERAKAA DAAARSVCHARTWFCFRKYDYVRRS
	$adD_{64–78}$	TERAKRRDRRRRSVC
	adDmut	TERAKAADAAARSVC
	$adD_{67–84}$	AKRRDRRRRSVCHARTWF
	$adD6_{9–84}$	RRDRRRRSVCHARTWF
	$adD_{69–83}$	RRDRRRRSVCHARTW
HIV-2	HIV-2 $Tat_{67–90}$	FLNKGLGIWYERKGRRRRTPKKTK
JC polyomavirus	Small $t_{115–134}$	MLKLRHRNRKFLRSSPLVWI
Human cathelicidin	LL37	LLGDFFRKSKEKIGKEFKRIVQRIKDFLRNLVPRTES
	$LL_{17–32}$	FKRIVQRIKDFLRNLV

Abbreviations: adD, adenodiaphorin; LadD, large adenodiaphorin.

Cytotoxicity assays

As previously described,[11] 3,000 cells were incubated for 24 hours with different concentrations of pharmacological agents. Cell cytotoxicity was analyzed by a colorimetric assay using MTT for adherent cells, as described by the manufacturer (Sigma).

The assays were performed in triplicate. We used Gen5 detection software (BioTek) for data capture and export into Excel. Histograms with error bars indicating the SD were created using Microsoft Excel software.

Bacterial strain and antibacterial susceptibility test

The *S. agalactiae* mutant NEM 316 ΔdltA strain, which is characterized by a complete absence of D-alanine due to the insertional inactivation of dltA, has been described previously.[18]

The minimum inhibitory concentrations (MICs) of each peptide were tested in Todd–Hewitt broth (THB) buffered with 50 mM HEPES in 96-well Costar polypropylene microplates (Costar, Cambridge, MA, USA) by a dilution method. Bacteria (10^6 CFU) were added in triplicate to wells containing increasing concentrations of the anti-microbial peptides. Plates were incubated for 24 hours at 37°C and then read (OD 600 nm) using a microplate reader (Synergy 2; BioTek) for bacterial growth. The MIC_{90} was considered to be the peptide concentration that inhibited 90% of growth.

Western blot analyses

As previously described,[11] exponentially growing cells (10^5 cells) were seeded overnight in 24-well culture cell plates, in a sub-confluent monolayer, prior to pharmacological treatments. For preparation of the extract, cells were rinsed in cold PBS, scraped, pelletized, and lysed in RIPA buffer 89900 supplemented with Halt Protease and Phosphatase Inhibitor Cocktail (78442; Thermo Scientific, Les Ulis, France), according to the manufacturer's instructions, and finally sonicated for 2 minutes at 50% pulse.

The protein concentration in each sample extract was quantified using a Bio-Rad Protein Assay (Bio-Rad Laboratories, Les Ulis, France). Lanes were loaded with the material corresponding to 20–40 µg of cell protein extract. The following primary antibodies were used: anti-phospho-AKT (Ser 473) (D9E) and anti-AKT (pan) (C67E7) (Cell Signaling Technology, Saint-Cyr-l'École, France), and anti-HP1γ (2MOD-1G6) (Euromedex). Goat peroxidase-labeled anti-rabbit IgG or horse peroxidase-labeled anti-mouse IgG antibodies

(Vector Laboratories) were used as secondary antibodies. Immunoreactivities were visualized using Pierce ECL Western Blotting Substrate and the myECL Imager (Thermo Scientific) and ImageJ 1.45s software (National Institutes of Health, USA, http://imagej.nih.gov/ij). After exporting into Excel, we used Microsoft Excel software 2016 for macOS for statistical analyses.

Results

LadD, a PTD encoded by the human ad2 E4orf4 protein, inhibits the phosphatidylinositol-3 (PI3)-kinase-dependent survival pathway of human U87G glioblastoma cells

The ad2 E4orf4$_{66–74}$ residues contain an arginine/lysine-rich motif (RAKRRDRRR) located inside the multifunctional E4orf4$_{64–95}$ domain that is involved in nuclear localization,[16] and is partially homologous to the HIV-1 TAT$_{47–57}$ cell-penetrating sequence (Tat cpp) (Table 2). This observation suggests that ad2 E4orf4 may be a new cell-penetrating protein. Consistent with this hypothesis, the use of chemical synthesis of peptides containing biotinylated ad2 E4orf4 sequences (detailed sequences shown in Table 2) indicated that the full-length wild-type E4orf4$_{1–114}$ polypeptide, the E4orf4$_{64–95}$ containing the E4orf4$_{66–74}$ cationic stretch, named large adenodiaphorin (LadD or LadD$_{64–95}$), and the shorter E4orf4$_{64–78}$ sequence, named adenodiaphorin (adD or AdD$_{64–78}$), can deliver streptavidin–HRP into U87G glioblastoma cells with similar cargo efficiencies to the HIV-1 Tat peptide (Figure 1, upper panel). Mutations either resulting from the deletion of the E4orf4$_{64–95}$ domain (Eorf4Δ$_{64–95}$) or involving (R→A) substitution within the LadD$_{64–95}$ sequence (LadDmut) or within the shorter adenodiaphorin adD$_{64–78}$ (adDmut) sequence suppressed this cargo effect.

In addition, both LadD and adD adenodiaphorin sequences displayed Tat-like kinetic cargo delivery properties (Figure 1, middle panel). Finally, as illustrated in Figure 1 (lower panel), E4orf4 as well as LadD and adD adenodiaphorin sequences also displayed similar Tat cargo delivery in DHF cells.

Furthermore, the cytotoxicity of LadD$_{64–95}$ and adD$_{64–78}$ penetrating peptides was investigated in U87G cells by the MTT assay. As shown in Figure 2 (upper panel), treatment of U87G cells with increasing amounts of LadD$_{64–95}$ for 24 hours resulted in a dose-dependent reduction in cell viability of the U87G glioblastoma cells. In contrast, no significant toxicity was detected in the presence of adD peptide. In addition, and in contrast to adD$_{64–78}$, LadD$_{64–95}$ inhibited AKT phosphorylation in U87G glioblastoma cells (Figure 2, lower panel). The two short penetrating sequences, AdD$_{64–78}$ and Tat$_{47–57}$ cpps, containing homologous nuclear localization signals,-[16,19] respectively, **RA*KRR*D*RRR*** in adenovirus E4orf4 adD and **RK*KRR*Q*RRR*** in HIV-1 Tat (single-letter amino acid code; basic residues are highlighted in bold type), displayed similar biological properties, such as cell penetration (Figure 1) without toxicity (Figure 2). In contrast, the larger adenodiaphorin LadD$_{64–95,}$ containing the previously described PP2A binding sequence required for cell toxicity,[14] inhibited survival of U87G cells. Finally, as shown in Figure 1 (upper panel), we confirmed that the suppression of cationic properties resulting from the

Table 2 Sequence of N-terminus biotinylated peptides used in this study

Acronym	Sequence
TATcpp	YGRKKRRQRRR
E4orf4	MVLPALPAPP VCDSQNECVG WLGVAYSAVV DVIRAAAHEG VYIEPEARGR LDALREWIYY NYY**TER**AKRR DRRRRSVCHA RTWFCFRKYD YVRRSIWHDT TTNTISVVSA HSVQ
E4orf4 Δ(64–95)	MVLPALPAPP VCDSQNECVG WLGVAYSAVV DVIRAAAHEG VYIEPEARGR LDALREWIYY NYY- - - - - - - - - - - - - - - - - SIWHDT TTNTISVVSA HSVQ
E4orf4$_{64–95}$ = LadD	**TER**AKRR DRRR RSVCHA RTWFCFRKYDYVRRS
E4orf4$_{64–78}$ = AdD	**TER**AKRR DRRR RSVC
LadDmut	**TER**AK**AA** D**AAA** RSVCHA RTWFCFRKYDYVRRS
adDmut	**TER**AK**AA** D**AAA** RSVC

Notes: Amino acid residues are expressed in one-letter conventional code. Residues involved in cell killing are in blue and residues involved in PP2A binding and cell killing are in red.[14] Mutations corresponding to R→A substitution are underlined.
Abbreviations: adD, adenodiaphorin; LadD, large adenodiaphorin.

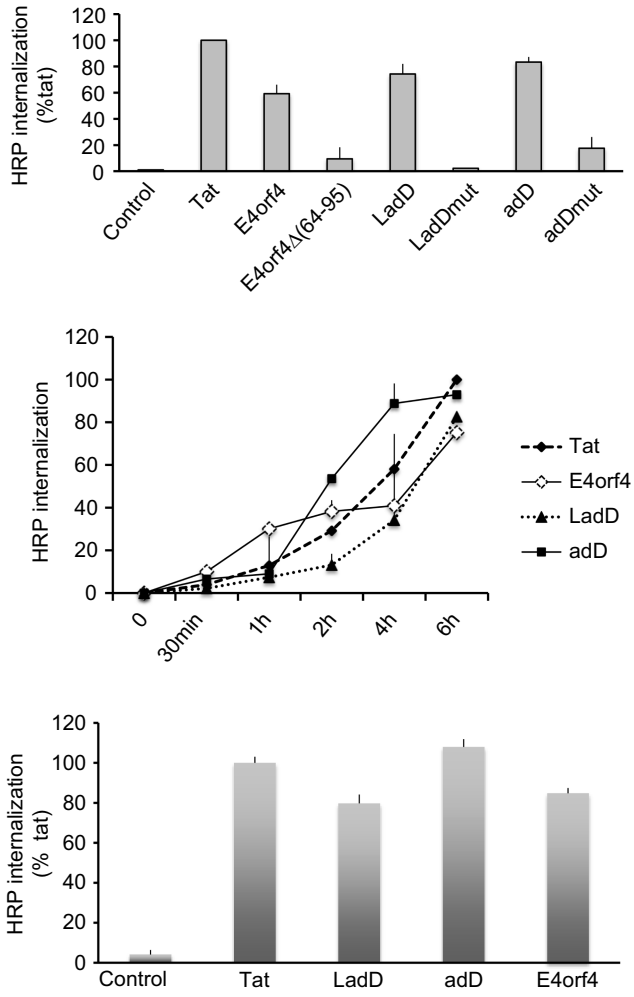

Figure 1 Effects of human adenovirus type 2 E4orf4 peptides on intracellular delivery of streptavidin–peroxidase in human U87G glioblastoma cells and in human DHF cells. Streptavidin–peroxidase coupled with 125 nM of biotinylated peptides was incubated at 37°C for 6 hours (upper panel) or 0–6 hours (middle panel) with U87G, and for 6 hours (lower panel) with DHF cells. Internalized complexes were visualized by a colorimetric test, OPD, as described previously.[11,13] HRP internalization of E4orf4 peptides is expressed as % of Tat-mediated HRP peptide (incubated for 6 hours) used as positive control. SD is shown for n=3. For negative control (Control), no peptide, no HRP, HRP alone, or cargo-inactive DPT-sh1 peptide (VKKKIKREIKI) was used, giving similar results. 6.88±0.96 ng and 4.29±0.82 ng of HRP, respectively, were internalized by 105 U87G (upper and middle panels) and by 105 DHF (lower panel) cells following 6-hour incubation with 125 ng of biotinylated-Tat peptide complexed with streptavidin–peroxidase.

Abbreviations: adD, adenodiaphorin; DHF, dermal human fibroblast; HRP, horseradish peroxidase; LadD, large adenodiaphorin; OPD, O-phenylenediamine dihydrochloride.

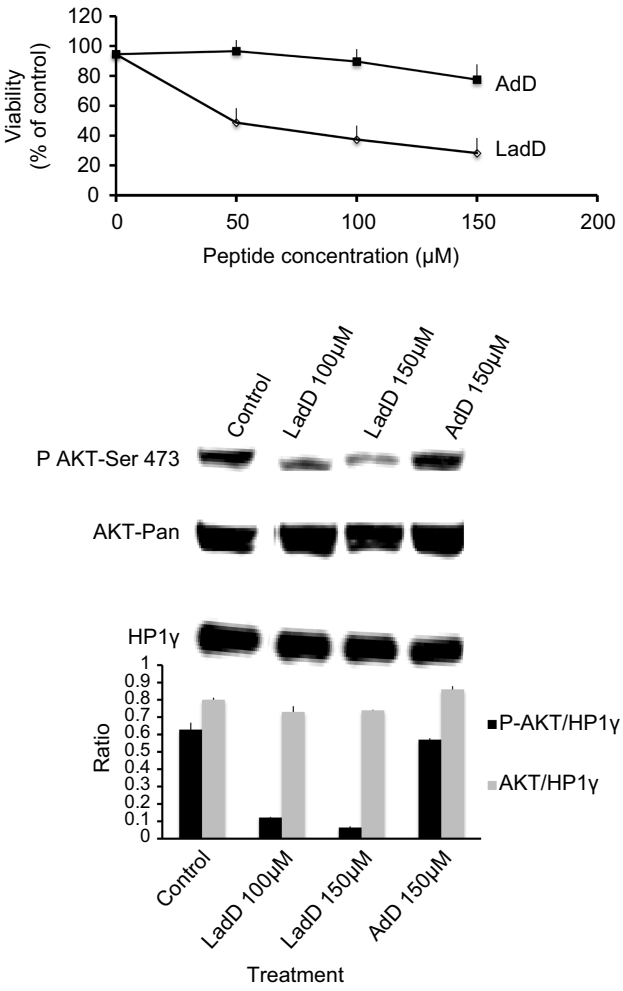

Figure 2 LadD inhibits a constitutively active PI3K/Akt survival pathway in U87G cells. Upper panel: cells were treated for 24 hours with LadD and adD peptides (0–150 μM), and cell viability was assessed by the MTT test (n=3). The lower panel shows a Western blot probed with the antibody specifically recognizing phosphorylated Akt-pSer473 (D9E rabbit mAb from cell signaling). The same blot was reprobed with the antibody specifically recognizing total Akt (C67E rabbit mAb from cell signaling) and with the antibody specifically recognizing HP1γ (2MOD-1G6 mouse mAb from Euromedex) that was used as loading control. Cells were untreated (control) or treated for 5 hours at 37°C with 100 μM or 150 μM of LadD, or with 150 μM of AdD peptides. The Western blot was quantified with ImageJ software, and AKT/HP1γ and pAKT/HP1γ normalized ratios, corresponding to the quotient of AKT or pAKT versus HP1γ expression, are illustrated in a histogram shown in the lower panel (n=3).

Abbreviations: adD, adenodiaphorin; DHF, dermal humanfibroblast; LadD, large adenodiaphorin; mAb, monoclonal antibody.

substitution of R with A in LadD (LadDmut) and adD (adDmut) also ablated cell penetration. Since we have previously demonstrated that both Akt basal constitutive activity and U87G survival are downregulated by specific PI3K/Akt pharmacological inhibitors,[20] these results indicate that $LadD_{64–95}$, but not $adD_{64–78}$, inhibited the constitutively active PI3K/Akt pathway required for the survival of U87G cells. In addition, although wild-type LadD and adD adenodiaphorin sequences have similar penetrating properties in U87G and in non-transformed DHFs (Figure 1, upper and lower panels), no toxicity was detected in DHF cells (not shown) where, in contrast to U87G, the PI3K/Akt survival pathway is not constitutively activated.[20]

Comparative analyses of the effects of human cathelicidin LL37 and several virally encoded peptides on growth of D-alanylated mutants of human pathogen group B *S. agalactiae* and on the survival of human U87G glioblastoma cells

In addition to HIV-1 Tat cpp, the arginine/lysine-rich motif (RAKRRDRRRR) localized in $LadD_{64–95}$ is also partially homologous to virally encoded arginine/lysine-rich motifs deduced from HIV-1 Vpr, HIV-2 Tat, and JC polyomavirus small t proteins, suggesting that the derived viral sequences may display common properties. In this regard, as mentioned in the Introduction, these virally encoded cationic sequences have similar physical characteristics to the anti-microbial LL37 molecule, suggesting that these sequences may behave as cathelicidin-like host defense molecules.[17] Therefore, to test this hypothesis, we comparatively investigated the anti-microbial activities of these viral peptides with the two human LL-37 and $LL_{17–32}$ molecules by monitoring the bacterial growth of *S. agalactiae* NEM 316 ΔdltA strain, a Gram-positive bacterial model that is highly sensitive to human LL37 cathelicidin.[18]

As shown in Table 3 (column 4), the MIC_{90} values of LL37, HIV-1 Vpr, adenodiaphorins, and JC polyomavirus small $t_{115–134}$ lie in a similar range (12.5–25 μM). The C-terminal $LL_{17–32}$ fragment is more efficient than the full-length LL37 (MIC_{90} 12.5 μM and 6.25 μM, respectively). In addition, and surprisingly, HIV-2 $Tat_{67–90}$ is slightly more active than $LL_{17–32}$ (MIC_{90} 3.1 μM and 6.25 μM, respectively). As expected, no antibacterial activity was observed with $Tat_{47–57}$ cpp, used as a negative control.

Table 3 Physical characteristics and antibacterial effects of cathelicidins LL37 and $LL_{17–32}$ and virally encoded cationic peptide sequences on *Streptococcus agalactiae* NEM 316 strains

Proteins : origin (cathelecdidin or viruses) acronym and sequences	Charges Net ° density	Basic/hydrophobic residues	*MIC_{90}
HCAP-18			
LL37	6 0.162	0.785	12.5μM
LLGDFFRKSKEKIGKEFKRIVQRIKDFLRNLVPRTES			
LL_{17-32} FKRIVQRIKDFLRNLV	4 0,250	0,62	6.25μM
HIV-1			
89.6 Vpr HFRIGCRHSRIGIIQQRRTRNG	8 0.363	1.60	25μM
Tat_{47-57}(cpp) YGRKKRRQRRR	9 0.346	-	No effect
HIV-2			
Tat_{67-90} FLNKGLGIWYERKGRRRRTPKKTK	9 0.375	1.66	3.1μM
Adenovirus 2			
$LadD_{64-95}$			
TERAKRRDRRRRSVCHARTWFCFRKYDYVRRS	11 0.343	2.00	12.5μM
adD_{64-78}			
TERAKRRDRRRRSVC	6 2.500	8	no effect
adD_{67-84}			
AKRRDRRRRSVCHARTWF	8 0.400	1.50	12.5μM
adD_{69-84}			
RRDRRRRSVCHARTWF	7 0.388	1.60	25μM
adD_{69-83}			
RRDRRRRSVCHARTW	7 0.410	2.00	25μM
JC PolyomaVirus			
$smallt_{115-134}$ MLKLRHRNRKFLRSSPLVWI	7 0.343	0.700	12.5μM

Notes: Amino acid residues are expressed in one-letter conventional code. Residues in red correspond to acidic residues (eg, D, E), residues in blue correspond to basic residues (eg, R, K, H), and residues in green correspond to hydrophobic uncharged residues (eg, F, I, L, M, V, W, A, P). °Charge density is calculated by dividing the net charge by the total number of amino acid residues (for detailed calculation see https:www.genescript.com/tools/peptide-properties-calculator). *The minimum inhibitory concentration (MIC; μM) of each peptide is an average of triplicate measurements performed by a dilution method in 96-well polypropylene microplates. The MIC_{90} was considered to be the peptide concentration that inhibited growth of 90% of the tested strains.

We also comparatively investigated the effect of human cathelicidin LL37 and virally encoded cationic peptides on survival of human U87G glioblastoma cells. We performed the MTT assay to monitor cytotoxicity of full-length LL-37 and C-terminal $LL_{17–32}$, also named FK16,[21] peptides in the U87G glioblastoma cell line. As shown in Figure 3 (upper panel), consistent with the previously described toxic effect in colon cancer cells,[21] both LL37 and $LL_{17–32}$ peptides provoke a similar and important reduction in the viability of U87G cells in a dose-dependent manner. Furthermore, as illustrated in Figure 3 (lower panel), we observed a dose-dependent reduction in U87G cell viability with $adD_{67–84}$ and $adD_{69–84}$, but not $adD_{69–83}$ (F84 deleted mutant). In addition, no significant toxicity was observed with HIV-2 $Tat_{67–90}$ and JC polyomavirus small $t_{115–134}$ treatments.

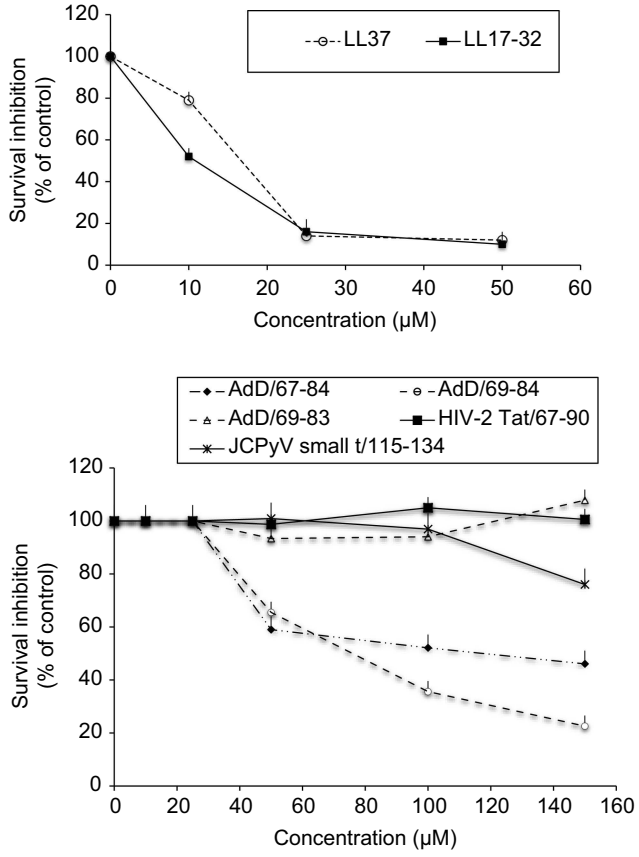

Figure 3 Effect of cathelicidin LL-37/$LL_{17–32}$, adenodiaphorin$_{67–84/69–84/69–83}$/deletion mutants, HIV-2 $Tat_{67–90}$, and JC polyomavirus small $t_{115–134}$ sequences, on viability of U87G glioblastoma cells. U87G cells were treated for 24 hours with the LL37 and $LL_{17–32}$ cathelicidin peptides (0–50 μM) (upper panel) or with virally encoded LadD deletion mutants ($AdD_{67–84}$, $AdD_{69–84}$, and $AdD_{69–83}$), HIV-2 $Tat_{67–90}$, and JC polyomavirus small $t_{115–134}$ cationic peptides (0–150 μM) (lower panel). Cell viability was assessed by the MTT test (n=3).
Abbreviations: adD, adenodiaphorin; LadD, large adenodiaphorin.

Discussion

The VQCS model predicts that multifunctional specific virally encoded cationic sequences may have the capacity to penetrate cells and to deregulate important human intracellular signaling pathways, such as PP2A-mediated pathways, but may also display LL37 cathelocidin-like antagonistic effects against multiple pathogens such as bacteria or viruses.[17] In this study, following the identification of ad2 E4orf4 cationic penetrating sequences, named adenodiaphorins, we tested the VQCS hypothesis by comparatively analyzing host defense properties of adenodiaphorins and some specific cationic sequences encoded by different viruses using two distinct biological models: U87G, a well-characterized cell tumor model; and a group B *S. agalactiae* NEM316 ΔdltA highly sensitive to LL37 cathelicidin.

Host defense properties of adenovirus E4orf4 adenodiaphorin penetrating sequences

Adenoviruses are non-enveloped double-stranded DNA viruses that can infect human tissues to provoke mild gastrointestinal and respiratory symptoms, and are often associated with pediatric patients.[22] The common species C adenoviruses (serotypes Ad1, Ad2, Ad5, and Ad6) infect more than 80% of the human population early in life and they also form latent infections in human lymphocytes.[23] In this study, we found that the full-length Ad2 $E4orf4_{1–114}$ sequence is a new cell-penetrating polypeptide and we demonstrated that the $E4orf4_{64–95}$/$LadD_{64–95}$ sequence, previously characterized as a multifunctional PP2A-binding domain and as a PP2A-dependent death sequence, is also a new PTD. In addition, we analyzed the potential host defense properties of the anti-microbial LL-37 and $LL_{17–32}$ cathelicidin peptides with specific virally encoded cationic peptides, including E4orf4 adenodiaphorin sequences, against both glioblastoma cells and *S. agalactiae* NEM 316 ΔdltA strain.

Using U87G glioblastoma cells, we demonstrated that LL-37, the only cathelicidin found in humans, and its shorter active fragment $LL_{17–32}$, are potent inhibitors of U87G glioblastoma cell survival. It has been reported that LL37 can mediate a dual role in tumorigenesis. First, as a positive factor, LL37 can promote the growth of ovarian,[24] lung,[25] and breast cancers.[26] Second, LL37 can promote tumor suppression in gastric cancer,[27] acute myeloid leukemia,[28] and lymphocytic leukemia.[29] Since U87G is

a highly radio-resistant glioblastoma cell line, our results suggest that $LL_{17–32}$ may be beneficial in the treatment of glioblastomas. Previous work established that $PP2A_1$ inactivates Akt and $PP2A_1$ inhibition activates tumor survival pathways associated with cancer progression.[30] Furthermore, we have previously reported that two PP2A activators, the immunosuppressant FTY720[20] and the peptide-mimetic DPT-Cog,[31] downregulated a constitutively active PI3K–Akt tumor survival pathway controlled by PP2A in radio-resistant U87G glioblastoma cells. The ad2 E4orf4 protein interacts with the regulatory Bα-subunit of $PP2A_1$ to specifically induce p53-independent death of human cancer cells.[14,32] In addition, the $E4orf4_{64–95}$ domain (here renamed $LadD_{64–95}$) is involved in cell penetration, PP2A and Src binding, nuclear localization, and cell death mediated by the viral E4orf4 protein.[14,16,32–34] In this regard, we found in this study that LadD inhibits the PI3K-dependent pathway required for survival of U87G cells. We also identified the shortest adenodiaphorin, $adD_{69–84}$, that inhibits U87G survival. The F84 deletion in $adD_{69–83}$, critically involved in $PP2A_1$ binding,[14] stops the inhibition of U87G cell survival mediated by the $adD_{69–84}$ RRDRRRRSVCHARTWF sequence. In contrast to LL37 and $LadD_{69–84}$, no toxicity was detected in the presence of HIV-2 $Tat_{67–90}$ and JC polyomavirus small $t_{115–134}$ cationic peptides.

Using *S. agalactiae* NEM 316 ΔdltA strain, in agreement with previous work,[18] we found that $LL_{17–32}$ displayed better antibacterial activity (MIC=6.25 μM) than LL37 (MIC=12.5 μM). These data clearly suggest that similarly to LL37, some virally encoded adenodiaphorin sequences could act as endogenous host defense peptides. In addition, we identify $adD_{69–84}$ as the shortest dual antitumor and antibacterial active adenodiaphorin. Our data are also consistent with a regulatory model based on two distinct host defense mechanisms mediated by adenodiaphorins. First, similarly to full-length E4orf4,[14,32] adenodiaphorins could kill tumor cells by interacting with the $PP2A_1$ target. Second, similarly to many anti-microbial cationic peptides, adenodiaphorins could kill their bacterial targets after interaction with anionic components of the bacterial membrane.[35] In agreement with this hypothetical model, in $adD_{69–83}$ the deletion of residue F84, required for both E4orf4-mediated PP2A binding and tumor cell death,[14] prevents U87G cell death induced by $AdD_{69–84}$ but retains the antibacterial effect. $adD_{69–84}$ and $adD_{69–83}$ have the same net charge of +7, suggesting that, similarly to LL37 and other CAMPs, adenodiaphorins could kill their bacterial targets by disrupting membrane integrity.[35] Together, these results suggest that, consistent with HIV-1 Vpr's biological effects, PP2A intracellular interaction in human cells and LL37-like membrane disruption in bacteria may represent a general property shared by some virally encoded sequences, including HIV-1 Vpr and adenodiaphorin molecules.

Antibacterial properties of HIV-1 Vpr, HIV-2 $Tat_{67–90}$, and JC polyomavirus small $t_{115\text{-}13\text{-}90}$ sequences

We have previously established that the cell-penetrating C-terminal domain of HIV-1 Vpr 89.6 isolate can interact with the structural A subunit of $PP2A_1$ to induce cell death.[13] In addition, antibacterial effects of C-terminal HIV-1 Vpr against *E. coli* have been previously reported.[15] Here, we found an anti-Gram-positive bacterial effect of the HIV-1 Vpr C-terminal sequence ($Vpr_{71–92}$) against *S. agalactiae* NEM 316 ΔdltA strain. In addition, consistent with our VQCS hypothesis, we found an antibacterial effect of HIV-2 $Tat_{67–90}$ and JC polyomavirus small $t_{115\text{-}134}$ cationic sequences against the same strain.

Conclusion

In this study, we found that ad2 E4orf4 is a cell-permeable protein containing a new $E4orf4_{64–95}$ PTD. We also demonstrated that, similarly to the unique human cathelicidin LL37 host defense peptide, $LadD_{64–95}$ and several virally encoded cationic sequences, including the C-terminus HIV-1 89.6 $Vpr_{77–92}$, shorter adenodiaphorins $AdD_{67–84}$/$AdD_{69–84}$/$AdD_{69–83}$, and HIV-2 $Tat_{67–90}$ and JC polyomavirus small $t_{115\text{-}134}$, displayed similar toxicity against Gram-positive *S. agalactiae* NEM316 ΔdltA strain. Finally, $LadD_{64–95}$, $AdD_{67–84}$, $AdD_{69–84}$, and cathelicidin LL37 and $LL_{17–32}$ peptides, also inhibit the survival of human U87G glioblastoma cells. HIV-1 Vpr peptides were previously identified in serum and in the cerebrospinal fluid of HIV-1-infected individuals.[36,37] In addition, given that E4orf4 protein can be detected late in the infectious cycle,[34] E4orf4 sequences may, similarly to HIV-1 Vpr, be liberated after the lysing of infected cells and circulate in biological fluids. Together, in agreement with the potential infective effects predicted by the VQCS hypothesis, our results suggest that the presence of virally encoded cationic peptides, such as adenodiaphorins and HIV-1 Vpr peptides, which could circulate in biological fluids, may define a new paradigm for a

potential virally mediated innate immunity. In addition, it is noteworthy that anti-biofilm effects and wound-healing properties of LL-37 have already been shown, suggesting that LL37, or its derivatives, could be used to develop new therapeutic strategies against biofilm-mediated infections to treat polymicrobially infected wounds through topical application.[38,39] Since the results in Table 3 clearly indicate that viral peptides work in the same LL37 concentration range, in accordance with the VQCS hypothesis,[17] we can postulate that some peptides containing virally encoded sequences may behave as LL37 derivatives and may be used against infected wounds.

Our results represent the first experimental data consistent with the VQCS hypothesis. Furthermore, in conjunction with future work involving other viruses, microbes, and parasites, the mimicry of host defense peptides of viral origin may represent a promising approach to design new therapeutic molecules with anti-infective and antitumor effects, as previously suggested with cellular host defense sequences.[40]

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Disclosure

The authors declare no conflicts of interest in this work.

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